

Short Report: A Prospective Evaluation of Real-Time PCR Assays for the Detection of *Orientia tsutsugamushi* and *Rickettsia* spp. for Early Diagnosis of Rickettsial Infections during the Acute Phase of Undifferentiated Febrile Illness

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Abstract. One hundred and eighty febrile patients were analyzed in a prospective evaluation of *Orientia tsutsugamushi* and *Rickettsia* spp. real-time polymerase chain reaction (PCR) assays for early diagnosis of rickettsial infections. By paired serology, 3.9% (7 of 180) and 6.1% (11 of 180) of patients were confirmed to have acute scrub or murine typhus, respectively. The PCR assays for the detection of *O. tsutsugamushi* and *Rickettsia* spp. had high specificity (99.4% [95% confidence interval (CI): 96.8–100] and 100% [95% CI: 97.8–100], respectively). The PCR results were also compared with immunoglobulin M (IgM) immunofluorescence assay (IFA) on acute sera. For *O. tsutsugamushi*, PCR sensitivity was twice that of acute specimen IgM IFA (28.6% versus 14.3%; McNemar's $P = 0.3$). For *Rickettsia* spp., PCR was four times as sensitive as acute specimen IgM IFA (36.4% versus 9.1%; $P = 0.08$), although this was not statistically significant. Whole blood and buffy coat, but not serum, were acceptable specimens for these PCRs. Further evaluation of these assays in a larger prospective study is warranted.

INTRODUCTION

Scrub and murine typhus, caused by *Orientia tsutsugamushi* and *Rickettsia typhi*, respectively, are important acute febrile illnesses in Thailand.^{1,2} Clinical diagnosis is difficult because early symptoms are similar to other common infections such as dengue, leptospirosis, and malaria.³ Laboratory confirmation is also difficult, conventionally requiring either culture of the infectious agent in cell monolayers at biosafety level-3,⁴ or paired serology (indirect immunofluorescence assay [IFA]) for the detection of rising antibody titers.⁵

MATERIALS AND METHODS

We prospectively evaluated two real-time polymerase chain reaction (PCR) assays for the rapid diagnosis of rickettsial infections using several blood fractions. These assays were designed for use on the JBAIDS instrument (Joint Biological Agent Identification and Diagnostic System; BioFire Diagnostics, Inc. (formerly Idaho Technology), Salt Lake City, UT): a ruggedized, field-portable real-time PCR system.

Specimens from 180 patients recruited into a study of fever of unknown origin on the Thailand-Myanmar border were included in the evaluation. Consenting patients' ≥ 5 years of age, with a febrile illness ($\geq 38^\circ\text{C}$) of ≤ 7 days duration and a negative malaria test, were eligible for inclusion. Venous blood (EDTA tube) was collected for PCR at enrollment and serum was collected at enrollment and Day 14 for IFA analyses. Blood specimens were aliquoted and stored at -80°C before testing. This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand (MUTM 2011-008-01) and Oxford Tropical Research Ethics Committee (OXTREC 42-10).

To compare the impact of use of different blood fractions in these PCRs, DNA was extracted from whole blood (adult patients only), buffy coat (all patients), and selected acute serum specimens (described below). Eight hundred microliters of whole blood and serum were extracted using the IT 1-2-3 Platinum Path purification kit (BioFire Diagnostics, Inc.) and 200 μL of buffy coat was extracted using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Elution volumes were 200 μL (whole blood/buffy coat) and 100 μL (serum).

Two JBAIDS-based TaqMan probe-based real-time PCR assays were designed for the specific detection of *O. tsutsugamushi* (47-kDa outer membrane protein gene) or *Rickettsia* spp. (17-kDa antigen gene). Primer and probe sequences for 17-kDa gene were modified slightly from previously published assays (see Reference 6) and optimized for use on the JBAIDS platform (R17KF_4: 5'-AAA CAA G GK CAN GGH ACA CTT CTT G-3', R17KR: 5'-AAG TAA TGC RCC TAC ACC TAC TC-3', and RProbeV2: 5'-6FAM-CCG AAT TGA GAA CCA AGT AAT GC-TAMRA-3'). The limit of detection was determined by testing the lowest level of spiked template that could be detected in at least 95% of specimens (i.e., 19 out of 20 replicates) and found to be 25 copies of target template per reaction. Both target assays were multiplexed with an internal inhibition control. The DNA extracts (20 μL /assay) were added to the freeze-dried reagent tubes along with 20 μL of reconstitution buffer and immediately tested in duplicate (individual reaction volume 20 μL ; DNA template volume 10 μL /reaction) on the JBAIDS instrument. Positive and negative controls were included in each PCR run. The JBAIDS instrument software automatically determined the specific operation of the instrument, i.e. times, temperatures, and number of PCR cycles, and determined a result for the duplicate reactions. Whole blood and buffy coat specimens were tested for all adult patients. For children, only buffy coat fractions were tested because there was insufficient whole blood/serum to be tested. If an adult patient tested positive for the *O. tsutsugamushi* or *Rickettsia* assay in whole blood or buffy coat, the corresponding serum

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specimen was then also tested. The PCR was repeated for specimens yielding uncertain results (discordant results between duplicate reactions) on first testing.

For comparison, three alternate rickettsial real-time PCR assays were run on buffy coat specimens, using the same DNA extracts as for the JBAIDS-based PCRs. These PCRs targeted 47-kDa outer membrane protein gene of *O. tsutsugamushi*,⁶ 17-kDa antigen gene of *Rickettsia* spp.,⁷ and *R. typhi* ompB,⁸ as previously described. A human RNaseP gene PCR was used as an internal control to confirm the absence of PCR inhibition and to monitor extraction efficiency.⁹ One microliter of DNA template was added into each 25 μ L PCR reaction. The PCR was done on an ABI 7500 Fast instrument (Life Technologies, Grand Island, NY).

All statistical data analyses were performed using STATA/SE 10.1 (StataCorp, College Station, TX). Diagnostic accuracy of the PCRs was calculated by comparing PCR results to the paired sera IFA results in 2 \times 2 cross tabulation using the "diagt" routine.¹⁰ The McNemar test was used to compare test sensitivities. Binomial exact confidence intervals were calculated throughout.

RESULTS

Using a \geq 4-fold rise in IFA immunoglobulin M (IgM) antibody titers between the paired acute and convalescent serum specimens to define acute infection,¹¹ 6.1% (11 of 180) of the patients were diagnosed with acute murine typhus and 3.9% (7 of 180) were with acute scrub typhus. Using a cut-off titer of 400 as evidence of acute infection as previously described,¹² the sensitivity and specificity of acute specimen IgM IFA were 14.3% (95% confidence interval [CI]: 0.4–57.9) and 97.7% (95% CI: 94.2–99.4) for scrub typhus, 9.1% (95% CI: 0.2–41.3) and 99.4% (95% CI: 96.7–100) for murine typhus (raw data not shown), respectively.

One hundred and four whole blood fractions and 180 buffy coat fractions were tested by JBAIDS PCR. Three and six corresponding serum fractions were tested for the *O. tsutsugamushi* and *Rickettsia* assay, respectively. The overall positivity of the *O. tsutsugamushi* and *Rickettsia* assays was 1.7% (3 of 180) and 2.2% (4 of 180), respectively, when any of the blood fractions gave a positive result.

The specificity of *O. tsutsugamushi* assay was 99.0% (95% CI: 94.6–100) using whole blood, 99.4% (95% CI: 96.8–100) using buffy coat, and 99.4% (95% CI: 96.8–100) when the results of all blood fractions were combined. The sensitivity of the assay using whole blood, buffy coat, and combined blood fractions was 50.0% (95% CI: 6.8–93.2), 28.6% (95% CI: 3.7–71.0), and 28.6% (95% CI: 3.7–71.0), respectively. Serum fractions did not correctly identify either of the patients with positive whole blood or buffy coat fractions. A total

TABLE 1
Comparison of two PCR assays for detection of *Orientia tsutsugamushi* DNA from blood*

Paired serology result (IgM IFA)	<i>O. tsutsugamushi</i> PCR (JBAIDS instrument)		47 kDa PCR (ABI instrument)	
	Negative	Positive	Negative	Positive
Not scrub typhus	172	1	172	1
Acute scrub typhus	5	2	5	2

*PCR = polymerase chain reaction; IFA = immunofluorescence assay; IgM = immunoglobulin M.

TABLE 2

Comparison of three PCR assays for detection of *Rickettsia* spp. DNA from blood*

Paired serology result (IgM IFA)	<i>Rickettsia</i> spp. PCR (JBAIDS instrument)			17 kDa PCR (ABI instrument)		ompB PCR (ABI instrument)	
	Negative	Positive	Uncertain	Negative	Positive	Negative	Positive
Not murine typhus	168	0	1	169	0	169	0
Acute murine typhus	8	3	0	9	2	9	2

*PCR = polymerase chain reaction; IgM = immunoglobulin M; IFA = immunofluorescence assay.

of seven patients had serologically confirmed acute scrub typhus infection by paired IgM IFA. Of these, three patients were children and only the buffy coat fraction was tested by PCR, none of which gave positive results. Of the four adult patients, two were positive by PCR in both whole blood and buffy coat fractions, but were negative in the serum fraction. There was perfect correlation between results obtained from whole blood and buffy coat fractions. One additional adult patient who had no evidence of acute scrub typhus infection by paired IgM IFA was positive for PCR in all blood fractions.

For *Rickettsia* assay, the specificity was 100% in all blood fractions, although as a consequence of the small number of serum specimens tested ($N = 6$), the 95% CI for the serum fraction result was wide (2.5–100). The sensitivity of the assay using whole blood, buffy coat, serum, and combined blood fractions was 30.0% (95% CI: 6.7–65.2), 27.3% (95% CI: 6.0–61.0), 40.0% (95% CI: 5.3–85.3), and 36.4% (95% CI: 10.9–69.2), respectively. A total of 11 patients had serologically confirmed positive acute murine typhus infection by paired IgM IFA. One patient was a child and only buffy coat fraction was tested by PCR, which had a negative result. Of the 10 adult patients, four patients were positive by PCR in whole blood and/or buffy coat fractions. There was poor correlation between the results obtained from whole blood and buffy coat fractions and the corresponding serum fraction.

For *O. tsutsugamushi*, PCR sensitivity was twice that of acute specimen IgM IFA (28.6% [95% CI: 3.7–71.0] versus 14.3% [95% CI: 0.4–57.9]; McNemar's $P = 0.3$). For *Rickettsia* spp., PCR was four times as sensitive as acute specimen IgM IFA (36.4% [95% CI: 10.9–69.2] versus 9.1% [95% CI: 0.2–41.3]; $P = 0.08$).

The comparison of five PCR assays for detection of *O. tsutsugamushi* and *Rickettsia* spp. DNA from blood are shown in Tables 1 and 2. Both *O. tsutsugamushi* PCR and 47-kDa PCR showed perfect concordant results; 1.67% (3 of 180) were positive for both PCRs (Table 1). For *Rickettsia* spp. PCR, 1.67% (3 of 180) was positive. One uncertain result was considered negative because there was no evidence for acute murine typhus by reference test (Table 2). However this result could represent a true infection by another spotted-fever group *Rickettsia* sp. 1.11% (2 of 180) were positive for 17-kDa PCR and the same specimens (1.11% [2 of 180]) were confirmed positive by ompB PCR.

CONCLUSION

We found that both of the *O. tsutsugamushi* and *Rickettsia* assays had high specificity and there was good correlation with the alternate PCR assays. The clinical sensitivity was low but

PCR was more sensitive than acute specimen IgM IFA. It is probable that a combination of both molecular and antibody-based detection assays would be the ideal panel to adequately cover all diagnostic window periods and might allow confirmation of infection quickly enough to be useful for patient management.^{13,14} A further evaluation of these real-time PCR assays in a larger prospective study is warranted.

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REFERENCES

1. Suttinont C, Losuwanaluk K, Niwatayakul K, Hoontrakul S, Intaranongpai W, Silpasakorn S, Suwancharoen D, Panlar P,

- Saisongkorh W, Rolain JM, Raoult D, Suputtamongkol Y, 2006. Causes of acute, undifferentiated, febrile illness in rural Thailand: results of a prospective observational study. *Ann Trop Med Parasitol* 100: 363–370.
2. McGready R, Ashley EA, Wuthiekanun V, Tan SO, Pimanpanarak M, Viladpai-Nguen SJ, Jesadapanpong W, Blacksell SD, Peacock SJ, Paris DH, Day NP, Singhasivanon P, White NJ, Nosten F, 2010. Arthropod borne disease: the leading cause of fever in pregnancy on the Thai-Burmese border. *PLoS Negl Trop Dis* 4: e888.
3. Ellis RD, Fukuda MM, McDaniel P, Welch K, Nisalak A, Murray CK, Gray MR, Uthaimongkol N, Buathong N, Sriwichai S, Phasuk R, Yingyuen K, Mathavarat C, Miller RS, 2006. Causes of fever in adults on the Thai-Myanmar border. *Am J Trop Med Hyg* 74: 108–113.
4. Luksameetanasan R, Blacksell SD, Kalambaheti T, Wuthiekanun V, Chierakul W, Chueasuwanchai S, Apiwattanaporn A, Stenos J, Graves S, Peacock SJ, Day NP, 2007. Patient and sample-related factors that effect the success of *in vitro* isolation of *Orientia tsutsugamushi*. *Southeast Asian J Trop Med Public Health* 38: 91–96.
5. Blacksell SD, Bryant NJ, Paris DH, Doust JA, Sakoda Y, Day NP, 2007. Scrub typhus serologic testing with the indirect immunofluorescence method as a diagnostic gold standard: a lack of consensus leads to a lot of confusion. *Clin Infect Dis* 44: 391–401.
6. Jiang J, Chan TC, Temenak JJ, Dasch GA, Ching WM, Richards AL, 2004. Development of a quantitative real-time polymerase chain reaction assay specific for *Orientia tsutsugamushi*. *Am J Trop Med Hyg* 70: 351–356.
7. Wright CL, Nadolny RM, Jiang J, Richards AL, Sonenshine DE, Gaff HD, Hynes WL, 2011. *Rickettsia parkeri* in gulf coast ticks, southeastern Virginia, USA. *Emerg Infect Dis* 17: 896–898.
8. Henry KM, Jiang J, Rozmajzl PJ, Azad AF, Macaluso KR, Richards AL, 2007. Development of quantitative real-time PCR assays to detect *Rickettsia typhi* and *Rickettsia felis*, the causative agents of murine typhus and flea-borne spotted fever. *Mol Cell Probes* 21: 17–23.
9. Emery SL, Erdman DD, Bowen MD, Newton BR, Winchell JM, Meyer RF, Tong S, Cook BT, Holloway BP, McCaustland KA, Rota PA, Bankamp B, Lowe LE, Ksiazek TG, Bellini WJ, Anderson LJ, 2004. Real-time reverse transcription-polymerase chain reaction assay for SARS-associated coronavirus. *Emerg Infect Dis* 10: 311–316.
10. Seed P, 2001. *DIAGT: Stata Module to Report Summary Statistics for Diagnostic Tests Compared to True Disease Status*. Boston, MA: Boston College Department of Economics.
11. Blacksell SD, Jenjaroen K, Phetsouvanh R, Tanganuchitcharnchai A, Phouminh P, Phongmany S, Day NP, Newton PN, 2010. Accuracy of rapid IgM-based immunochromatographic and immunoblot assays for diagnosis of acute scrub typhus and murine typhus infections in Laos. *Am J Trop Med Hyg* 83: 365–369.
12. Coleman RE, Sangkasuwan V, Suwanabun N, Eamsila C, Mungviriyaya S, Devine P, Richards AL, Rowland D, Ching WM, Sattabongkot J, Lerdthusnee K, 2002. Comparative evaluation of selected diagnostic assays for the detection of IgG and IgM antibody to *Orientia tsutsugamushi* in Thailand. *Am J Trop Med Hyg* 67: 497–503.
13. Paris DH, Blacksell SD, Nawtaisong P, Jenjaroen K, Teeraratkul A, Chierakul W, Wuthiekanun V, Kantipong P, Day NP, 2011. Diagnostic accuracy of a loop-mediated isothermal PCR assay for detection of *Orientia tsutsugamushi* during acute scrub typhus infection. *PLoS Negl Trop Dis* 5: e1307.
14. Koh GC, Maude RJ, Paris DH, Newton PN, Blacksell SD, 2010. Diagnosis of scrub typhus. *Am J Trop Med Hyg* 82: 368–370.